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THE C-MYC CODING REGION DETERMINANT-BINDING  
PROTEIN (CRD-BP) AND ITS NUCLEIC ACID SEQUENCE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. provisional  
5 application Serial No. 60/077,372 filed March 9, 1998.  
Serial No. 60/077,372 is incorporated by reference as if  
herein set forth completely.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH  
OR DEVELOPMENT

10 This invention was made with United States  
government support awarded by the following agencies:  
NIH Grant No(s): CA63676; CA07175; CA23076. The United  
States has certain rights in this invention.

BACKGROUND OF THE INVENTION

15 The c-myc protein is a member of the  
helix-loop-helix/leucine zipper (HLH/LZ)<sup>1</sup> family of  
transcription factors that forms heterodimers with Max

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<sup>1</sup>The abbreviations used herein are: HLH/LZ, helix-loop-helix/leucine zipper; AURE, AU-rich element; UTR, untranslated region; CRD, coding region determinant; CRD-BP, coding region determinant-binding protein; DTT, dithiothreitol; EGTA, ethylene glycol bis(2 aminoethyl ether)-N,N' (tetraacetic acid); PMSF, phenylmethyl-sulfonylfluoride; S130, post-polysomal supernatant; SDS, sodium dodecyl sulfate; RSW, ribosomal salt wash; PCR, polymerase chain reaction; bp, base pairs; EST, Expressed Sequence Tags; RACE, rapid amplification of cDNA ends; BAC, Bacterial Artificial chromosome; GCG, Genetics Computer Group; IP, immunoprecipitation; mRNP, messenger ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; HRP, horseradish peroxidase; HSP-90, heat shock protein-90; MOPS, morpholinepropanesulfonic acid; KH, K homology; ORF, open reading frame; FMR, familial mental retardation; FMRP, FMR RNA-binding protein; hKOC, human KH domain protein overexpressed in human cancer; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescent.

(1-3). In general, trans-activating Myc:Max heterodimers are found in proliferating cells, while trans-repressing Mad:Max heterodimers are found in differentiated cells. The *c-myc* protein level influences cell proliferation, differentiation, and neoplastic transformation, presumably by affecting the balance between Myc:Max and Mad:Max heterodimers (4). When *c-myc* protein is overexpressed or is induced at inappropriate times, this balance is perturbed, and cell proliferation and differentiation are disrupted. For example, *c-myc* overexpression prevents or delays cell differentiation (5, 6). It also blocks serum-starved cells from entering the G<sub>0</sub> phase of the cell cycle and instead induces them to undergo apoptosis (7). *c-myc* overexpression is also implicated in tumor formation in experimental animals and in human patients with Burkitt's lymphoma (8, 9). These and other deleterious consequences of aberrant *c-myc* expression highlight the importance of understanding all aspects of *c-myc* gene regulation.

The *c-myc* protein is regulated by phosphorylation, protein:protein interactions, and changes in its half-life (10-12). *c-myc* mRNA levels are regulated transcriptionally and post-transcriptionally, and changes in *c-myc* mRNA stability can result in large fluctuations in *c-myc* protein levels. The *c-myc* mRNA half-life is normally only 10 to 20 minutes but can be prolonged 3- to 6-fold when necessary. For example, *c-myc* mRNA is relatively stable in replicating fetal rodent

hepatocytes, which produce abundant *c-myc* mRNA. It is far less stable in non-growing adult hepatocytes, which contain little or no *c-myc* mRNA (13, 14). However, it is up-regulated and stabilized several-fold when adult  
5 hepatocytes replicate following partial hepatectomy (15, 16).

Two *cis*-acting sequence elements in *c-myc* mRNA contribute to its intrinsic instability and perhaps also to its post-transcriptional regulation: an AU-rich  
10 element (AURE) in the 3'-untranslated region (3'-UTR) and a 180 nucleotide coding region determinant (CRD). The CRD encodes part of the HLH/LZ domain and is located at the 3' terminus of the mRNA coding region. Four observations indicate how the *c-myc* CRD functions  
15 independently of the AURE to affect *c-myc* mRNA expression. (i) *c-myc* mRNA lacking its CRD is more stable than wild-type *c-myc* mRNA (17-20). (ii) The CRD is required for the post-transcriptional down-regulation of *c-myc* mRNA that occurs when cultured myoblasts fuse to  
20 form myotubes (20, 21). (iii) Inserting the *c-myc* CRD in frame within the coding region of  $\beta$ -globin mRNA destabilizes the normally very stable  $\beta$ -globin mRNA (22). (iv) The *c-myc* CRD is necessary for up- and down-regulating *c-myc* mRNA levels in transgenic mice  
25 undergoing liver regeneration following partial hepatectomy (13, 15, 16, 23-25). In summary, the *c-myc* CRD influences *c-myc* mRNA stability in animals and in cultured cells.

We have investigated *c-myc* mRNA stability and the function of the CRD using a cell-free mRNA decay system that includes polysomes from cultured cells. The polysomes contain both the substrates (mRNAs) for decay and at least some of the enzymes and co-factors that affect mRNA stability. Polysomes are incubated for different times in an appropriate buffer system, and the decay rates of polysomal mRNAs such as *c-myc* are monitored by hybridization assays. This system reflects many aspects of mRNA decay in intact cells (26-29). For example, mRNAs that are unstable in cells are also relatively unstable *in vitro*; mRNAs that are stable in cells are stable *in vitro* (26). In standard reactions, the polysome-associated *c-myc* mRNA was degraded rapidly in a 3' to 5' direction, perhaps by an exonuclease (29). An alternative decay pathway became activated when the reactions were supplemented with a 180 nucleotide sense strand competitor RNA corresponding to the *c-myc* CRD. This CRD RNA induced endonucleolytic cleavage within the *c-myc* CRD, resulting in an 8-fold destabilization of *c-myc* mRNA (30). These effects seemed to be specific for *c-myc*. Other competitor RNAs did not destabilize *c-myc* mRNA, and *c-myc* CRD competitor RNA did not destabilize other mRNAs tested.

Based on these observations, we hypothesized that a protein was bound to the *c-myc* CRD. We further suggested that this protein shielded the CRD from endonuclease attack, that the CRD competitor RNA titrated the protein

off of the mRNA, and that the unprotected *c-myc* CRD was then attacked by an endonuclease. Consistent with this model, we detected a protein that binds strongly *in vitro* to a *c-myc* CRD <sup>32</sup>P-RNA probe (30). This protein, the  
5 *c-myc* coding region determinant-binding protein (CRD-BP), was subsequently purified to homogeneity (31). We then found that the CRD-BP is developmentally regulated, being expressed in fetal and neonatal rats but not in adult animals (32).

10 SUMMARY OF THE INVENTION

In the Examples below, we report the cloning of the mouse CRD-BP cDNA, a novel member of an RNA-binding protein family. We also show that the CRD-BP can bind to ribosomes *in vitro* and that most of the CRD-BP in cell  
15 extracts is located in the cytoplasm and is associated with polysomes and ribosomes. These observations are consistent with a role for the CRD-BP in shielding polysomal *c-myc* mRNA from endonucleolytic attack, which means that the CRD-BP helps to preserve *c-myc* mRNA and  
20 allows it to be used to make c-MYC protein. We believe that blocking CRD-BP expression might result in the very rapid destruction of *c-myc* mRNA and subsequent depletion of c-MYC protein from the cell.

We have also shown that the CRD-BP is abundantly  
25 expressed in cancer cell lines grown in the laboratory as well as in fetal tissues from rodents (32). In contrast, the CRD-BP is undetectable in tissues from adult rodents

(32). We believe that these latter observations may be consistent with the idea that the CRD-BP is an oncofetal protein--that is, a protein that is expressed in the fetus and in cancer cells in post-natal life but is not  
5 expressed in normal (non-cancerous) tissues in post-natal life. If so, then the CRD-BP should be present in cancer tissues but not in normal tissues in post-natal life.

Specific, restricted expression of the CRD-BP in cancerous tissues could mean that the CRD-BP is a  
10 potential diagnostic/prognostic marker for human cancer. Moreover, since the CRD-BP seems to protect *c-myc* mRNA from being destroyed rapidly, and since c-MYC protein is essential for cell growth, then eliminating the CRD-BP from cancer cells could lead to the cessation of their  
15 growth or even to their death.

The present invention is a method of diagnosing the presence or absence of cancer in a human patient comprising the steps of examining patient tissue for the CRD-BP expression levels and comparing that expression  
20 level with a control or examining patient serum for antibody against the CRD-BP and comparing that antibody level with that of normal controls (preferably age-matched and sex-matched). Preferably, the control for the CRD-BP expression level in tissues is a non-cancerous  
25 tissue from the same source as the test tissue. For example, a breast assay would preferably have a breast tissue control. In a preferred embodiment of the present invention, the cancer is selected from the group



consisting of breast cancer, colon cancer and pancreatic cancer.

In another preferred embodiment of the present invention, the detection of CRD-BP comprises the step of  
5 homogenizing biopsy tissue and obtaining a crude protein extract. One would then examine that extract for the CRD-BP level.

The present invention is also a quantitative method of determining the stage of cancer in a human patient  
10 comprising the step of examining patient tissues for the CRD-BP expression level and correlating that expression level with the disease prognosis.

The present invention is also a method of inhibiting cancer cell growth comprising the step of eliminating or  
15 lowering the level of CRD-BP in the cancerous cells.

It is an advantage of the present invention that a method of diagnosing human cancers is disclosed.

It is another advantage of the present invention that a method of inhibiting cancer cell growth is  
20 disclosed.

Other objects, advantages and features of the present invention will become apparent after one of skill in the art has examined the specification, claims and drawings.

25 BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1. Mouse CRD-BP cDNA and predicted protein sequence (SEQ ID NOs:1 and 2, respectively). Peptide

sequences resembling nuclear localization and nuclear export signals are denoted by the single underline and the overlines, respectively. Peptide sequences resembling the RGG box and the KH domains are denoted by the box and the double underlines, respectively. An asterisk indicates the translation termination site, and the polyadenylation signal is single underlined. We have not demonstrated conclusively that the translation start site indicated in the figure is the correct or the only start site. The 5'-UTR might be incomplete, since the transcription start site has not been mapped.

Fig. 2. CRD-BP alignments with various consensus sequences in RNA binding proteins (SEQ ID NOs:3-30). Shown are alignments of the mouse CRD-BP (mCRD-BP) to the RGG domains (A) (SEQ ID NOs:3-9) nuclear export signals (B) (SEQ ID NOs:10-16), and KH domains (C) (SEQ ID NOs:17-30) of other RNA-binding proteins. Referring to Fig. 2, boxed residues indicate identity with or conservation to the consensus sequence residue. The Genbank accession numbers of the proteins are as follows: hKOC, U97188; hnRNPK, S74678; fibrillarin, X56597; nucleolin, M60858/J05584; FMRP, S65791; Rev, X58781.

Fig. 3. Immunoblotting assay showing co-migration of recombinant and cell derived CRD-BP. Ribosomal salt wash (RSW) was prepared from K562 and NIH/3T3 cell polysomes and from polysomes isolated from reticulocyte transcription/translation reactions programmed with CRD-BP DNA or with vector DNA. Approximately  $7.5 \times 10^5$

cell equivalents of K562 or NIH/3T3 RSW or 3% of the RSW recovered from a 50  $\mu$ l translation reaction were electrophoresed in a 10% SDS-PAG and transferred to a membrane, which was incubated with anti-CRD-BP IgY antibody and then with HRP-conjugated anti-IgY antibody. The signal was developed with Supersignal chemiluminescent reagents. The locations of the CRD-BP and a cross-reacting protein (p85) are indicated. The locations of prestained molecular mass markers are shown on the right in kDa.

Fig. 4. Gel retardation assay showing specific binding of recombinant CRD BP to *c-myc* CRD RNA. (A) RSW was prepared from K562 cell polysomes and from transcription/translation reactions programmed with CRD-BP cDNA, luciferase cDNA (Luc), or vector DNA. Equivalent volumes (2  $\mu$ l) of each RSW were incubated with 50,000 cpm of synthetic *c-myc* CRD  $^{32}$ P-RNA. RNA/protein complexes were separated from free (unbound) probe by electrophoresis in a 6% nondenaturing PAG. "None" indicates a gel retardation reaction to which no protein was added. The positions of CRD-BP/CRD complexes (Bound) and of unbound (Free) RNA are indicated on the left. (B) Competition assay. The indicated RSW was incubated with *c-myc* CRD  $^{32}$ P-RNA in the presence or absence of buffer (None) or a 200-fold molar excess of unlabeled synthetic *c-myc* CRD RNA or  $\beta$ -Globin RNA. RNA/protein complexes were then separated in a 6% nondenaturing PAG. The

positions of CRD-BP/CRD complexes (Bound) and of unbound (Free) RNA are indicated on the left.

Fig. 5. Co-fractionation of recombinant CRD-BP with reticulocyte ribosomes and ribosomal subunits.

5 Radiolabeled recombinant CRD-BP (filled circles) and luciferase (LUC; unfilled circles) were synthesized in separate reticulocyte translation assays. Each extract was then fractionated by sedimentation through a 20-40% linear sucrose gradient. Equivalent amounts of each  
10 gradient fraction were analyzed for radiolabeled protein by electrophoresis in a 10% SDS-PAG and quantitation in the Phosphorimager. The quantity of CRD-BP and luciferase is given in arbitrary units. The locations of ribosomal subunits, monosomes, and polyribosomes were  
15 determined by measuring A260 and by electrophoresing a portion of each fraction in an agarose gel, to identify 18S and 28S rRNAs.

Fig. 6. Co-fractionation of endogenous CRD-BP with K562 cell polysomes and lack of CRD-BP in nuclei.

20 Subcellular fractions were prepared from exponentially growing K562 cells (Experimental Procedures). Equal cell equivalents ( $6 \times 10^5$ ) of each fraction were separated in a 10% SDS-PAG, transferred to a nitrocellulose membrane, and incubated with either (A) anti-CRD-BP IgY or (B)  
25 anti-HSP-90 IgG, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive proteins were visualized using ECL

reagents. The positions of molecular mass markers are indicated on the left in kDa.

Fig. 7. Co-fractionation of the CRD-BP with ribosomal subunits from K562 cells. Polysomes from exponentially growing K562 cells were resuspended in buffer and then incubated in 20 mM EDTA to dissociate ribosomal subunits (60S and 40S) from each other and from mRNP. An aliquot of the subunits was centrifuged in a linear 5-30% sucrose gradient containing EDTA. Fraction 1 is the top of the gradient, fraction 18 is the last gradient fraction, and fraction 19 is the pellet resuspended from the bottom of the centrifuge tube. Panel A: absorbance of each fraction at 260 nm. Panel B: RNA isolated from an aliquot of each fraction was electrophoresed in a 1% agarose gel, which was stained with ethidium bromide and photographed under UV light. The positions of the 28S and 18S rRNAs from the large and small ribosomal subunits, respectively, are noted on the left. Panel C: An aliquot of each fraction was analyzed by immunoblotting using anti-CRD-BP IgY. Immunoreactive proteins were visualized using ECL reagents. The positions of molecular mass markers are indicated in kDa on the left. The CRD-BP and the cross-reacting p85 are noted on the right.

Fig. 8. Co-fractionation of the CRD-BP with 60S ribosomal subunits as determined by immunoprecipitation with anti P-protein antibody. An aliquot of EDTA dissociated K562 cell polysomes was incubated with

anti-P protein antibody (I) or with normal human serum (N). Antibody-antigen complexes were immunoprecipitated (IP'd), and IP'd proteins were immunoblotted and analyzed using anti-P protein IgG (panel A) or anti-CRD-BP IgY (panel B). Immunoreactive proteins were visualized using ECL reagents. The locations of the P proteins ( $P_0$ ,  $P_1$  and  $P_2$ ) and the CRD-BP are indicated on the right. The positions of prestained molecular mass markers are indicated in kDa on the left. Heavy chain indicates cross-reactivity with the IgG heavy chain on the membrane.

#### DETAILED DESCRIPTION OF THE INVENTION

##### A. In General

The half-life of c-myc mRNA is regulated when cells change their growth rates or differentiate. Two sequences within the c-myc mRNA molecule determine its half-life, one in the 3'-untranslated region, the other in the coding region. A cytoplasmic protein, the coding region determinant-binding protein (CRD-BP), binds *in vitro* to the c-myc coding region stability determinant.

Based on observations using a cell-free mRNA decay system, we propose that the CRD-BP, when bound to the mRNA, shields the mRNA from endonucleolytic attack and thereby prolongs the mRNA half-life. Here we describe the cloning and further characterization of the mouse CRD-BP, a 577 amino acid protein containing four hnRNP K-homology domains, an RGG RNA-binding domain, and

nuclear import and export signals. The CRD-BP is similar to a human protein overexpressed in certain human cancers. Recombinant mouse CRD-BP binds specifically to *c-myc* CRD RNA *in vitro* and reacts with antibody against  
5 human CRD-BP. *In vitro* translated CRD BP binds to ribosomes in the absence of *c-myc* mRNA, and much of the CRD-BP in cell lysates is associated with ribosomes.

We also describe below proposed methods for the present invention. In one embodiment, we propose a  
10 method of diagnosing the presence or absence of cancer in a human patient comprising the steps of examining patient tissue for the CRD-BP expression levels and comparing that result with a control sample and/or examining patient serum for antibody against the CRD-BP and  
15 comparing that antibody level with that of normal controls (preferably age-matched and sex-matched). Preferably, the control sample for the CRD-BP expression level in tissues is a non-cancerous tissue from the same source. For example, one would compare the CRD-BP levels  
20 of a test breast tissue sample with the CRD-BP levels of breast tissue known to be non-cancerous.

This examination may take the form of examining a crude protein extract for the CRD-BP level, preferably by two antibody sandwich assay, antigen competition assay,  
25 antibody capture assay, or by immunoblotting of the crude protein extract with an antibody to CRD-BP. One may also examine the cells in the tissue samples directly for the presence or absence of CRD-BP via immununological methods

involving probing a tissue section with an antibody to CRD-BP or via *in situ* hybridization methods involving probing a tissue section with a nucleic acid probe specific for the CRD-BP.

5           In another embodiment, the present invention is a method of determining cancer disease prognosis. One would examine the CRD-BP expression levels in a patient tissue sample and correlate these CRD-BP levels with disease prognosis.

10           The present invention is also the use of CRD-BP in immunological assays to identify and quantify anti-CRD-BP antibodies in patient sera. Preferably, one would use recombinant CRD-BP in standard immunological assays. The present invention is also the use of anti-CRD-BP  
15           antibodies to identify and quantify the CRD-BP itself in serum from cancer patients.

We expect to find that certain expression levels of CRD-BP can be directly correlated with, and are therefore predictive of, certain cancers.

20           We also propose a method of inhibiting cancer cell growth by eliminating or lowering the level of CRD-BP from the cancerous cells. Preferably, this method is either by providing the cell with competitor RNA or by use of an inhibitor that blocks CRD-BP binding to the c-  
25           myc mRNA CRD.

By "CRD-BP" we preferably mean the protein as described herein at SEQ ID NO:2 and in Ref. 30, 31 and 32 below.



One typical way to obtain a CRD-BP antibody would be to make large amounts of recombinant CRD-BP in either bacterial cells, yeast cells or baculovirus-infected insect cells. This protein is then injected into  
5 rabbits, sheep or goats to make a polyclonal antibody. Epitope-specific antibodies can also be made by using synthetic peptides (8-15 amino acids) as the immunogen. These are routine techniques known to those of skill in the art.

10 B. Detecting the CRD-BP in Clinical Samples

We have hypothesized that the CRD-BP might be an oncofetal protein. This hypothesis is based on our findings that the CRD-BP is expressed in fetal rat tissues but not in normal adult rat tissues. It is also  
15 expressed in tissue culture cell lines, which are neoplastic.

We show below in the Examples that the CRD-BP is significantly more abundant in tumor tissue than in a normal adult tissue. Therefore, we envision that the  
20 presence of the CRD-BP in biopsy specimens indicates that the specimens contain tumor cells. We envision that the presence of the CRD-BP is indicative of neoplasia and would be a prognostic and diagnostic indicator.

There are many possible CRD-BP detection schemes.  
25 The best scheme will depend on the following variables: the amount of CRD-BP expressed in the tumor tissue, the specificity and avidity of the antibodies for the CRD-BP, and the extent of cross-reactivity of the antibodies with

other proteins besides the CRD-BP. Below is an outline of several possible detection schemes.

It is probably best to ensure that the antibodies are specific for the CRD-BP. We can do so by making  
5 antibodies against CRD-BP peptides or by using monoclonal antibodies that, on Western blots, react only with the CRD-BP and not with any other cellular proteins..

10 1. Detection of the CRD-BP using protein extracts:  
Biopsy tissue would be homogenized and a crude protein extract would be prepared (Proposed).

a. Exemplary Detection schemes in which antigen or antibody is bound to a solid support.  
15 i. Two antibody sandwich assay: A monoclonal antibody recognizing one CRD-BP epitope is bound to a solid support such as a microtiter well. The sandwich assay would also work with two polyclonal antibodies, as long as each antibody was against a different epitope in  
20 the CRD-BP. An extract of the tissue is added, and CRD-BP in the extract is permitted to bind to the antibody. Then a second monoclonal recognizing a different CRD-BP epitope is added. The second antibody can be labeled with  $^{125}\text{I}$  or  $^3\text{H}$ . Then, the amount of labeled antibody  
25 bound will provide a measure of the amount of CRD-BP attached to the first antibody.

Alternatively, a tagged secondary antibody can be used for quantitation. This secondary antibody can be tagged with an enzyme such as horseradish peroxidase or  
30 with a probe such as biotin. The amount of bound secondary antibody is then detected by standard assays

and is a measure of the amount of CRD-BP in the tissue extract.

ii. Antigen competition assay: Anti-CRD-BP antibody is bound to a solid support such as a microtiter well. The tissue extract is then mixed with purified, radiolabeled CRD-BP. If the tissue contains sufficient CRD-BP, this CRD-BP will compete with the labeled CRD-BP for binding to limiting antibody. Thus, the amount of CRD-BP in the extract will be inversely proportional to the amount of labeled CRD-BP bound to the microtiter well. We know the nucleic acid sequence of the human CRD-BP coding region. Therefore, we should be able to prepare highly purified, radiolabeled CRD-BP using bacteria, yeast, or insect cells.

Prokipcak, et al. (ref. 31) discloses one method of purification of CRD-BP. We also envision an easier purification scheme that exploits added epitopes. Instead of making unmodified CRD-BP in bacteria, yeast, or baculovirus-infected cells, we could use molecular techniques to design a CRD-BP complementary DNA that would generate an "epitope-tagged" CRD-BP. We could express the tagged CRD-BP in cells and then purify the CRD-BP in a single affinity step that exploits the tag to separate CRD-BP from all the other cell proteins.

iii. Antibody capture assay: The tissue extract is bound to a microtiter well. Antibody is added, and the amount of antibody bound is determined. The antibody can be labeled or unlabeled. If it is

unlabeled, the amount bound is determined indirectly, using anti-antibody antibodies and detecting them by peroxidase or biotin labeling, as described above.

5           b.   Exemplary Detection of the CRD-BP by  
              Immunoblotting (Western Blotting)

Tissue extract is electrophoresed in a denaturing gel, and the proteins are transferred to a nitrocellulose or PVDF membrane. The membrane is then probed with anti-CRD-BP antibody, and the amount of antibody bound is  
10   determined by any of a variety of detection techniques using tagged anti-antibody antibodies. The disadvantage of Western blotting is that it is more time-consuming than assays in which the extract protein or the antibody is bound to a solid support. The advantage is that  
15   specific interactions are more readily discerned, and artifacts are eliminated. The presence of the CRD-BP in a Western blot is indicated by a band at the ~68 kilodalton region of the gel.

We envision that the assay might be simplified to  
20   the point that a dipstick or colorimetric assay could be used.

2.   Detection of CRD-BP in cells by immunohistochemistry

25           In a typical method, the biopsy tissue is cut into a thin section and fixed and then analyzed using standard immunohistochemical techniques. The detection system will depend on the amount of CRD-BP in the tissue. Although this technique is more time-consuming than  
30   techniques using tissue extracts, immunohistochemistry

can identify rare abnormal cells. For example, a biopsy specimen might contain primarily normal cells with only small patches of neoplastic cells. If the neoplastic cells express the CRD-BP, then they might be visualized  
5 by immunohistochemistry using CRD-BP-specific antibodies.

3. Detection of CRD-BP in cells by in situ hybridization

In a typical method, the biopsy tissue is cut into a thin section and fixed and then analyzed using standard  
10 in situ hybridization techniques with a CRD-BP DNA or RNA probe. As is the case with immunohistochemistry, an advantage of the in situ hybridization technique is the ability to detect rare cancerous cells in the midst of a majority of normal cells.

15 C. Detecting CRD-BP or CRD-BP Antibodies in Patient Sera

The CRD-BP is a cytoplasmic protein. Therefore, it should not be exposed to immune cells under most conditions. However, if it is overexpressed in human  
20 tumor cells, and if these cells undergo lysis or the protein for whatever reason leaks out of the cells, the CRD-BP itself might be detected in patient serum, and/or antibodies to the CRD-BP might arise in patients with tumors. Detecting the CRD-BP or such antibodies in a  
25 small amount of patient serum would then provide a rapid and convenient screen for cancer. The previous section outlined methods for detecting the CRD-BP. Strategies to detect anti-CRD-BP antibodies might exploit techniques similar to those for detecting the CRD-BP itself in

extracts from biopsy material. There are many ways for detecting antibodies. Some of the techniques that would be suitable for detecting anti-CRD-BP antibodies in patient serum are summarized below.

5           i.    Two Antibody Sandwich Assay

The CRD-BP itself will be made in bacterial, yeast or insect cells using standard techniques. This recombinant CRD-BP will then be bound to a solid support such as a microtiter well. Patient serum is added, and  
10 anti-CRD-BP antibody in the serum is permitted to bind to the CRD-BP. The plates are then washed extensively, and a second anti-human serum is added. The second antibody can be labeled with <sup>125</sup>I or <sup>3</sup>H or with a fluorescent tag. Then, the amount of labeled antibody bound will provide a  
15 measure of the amount of anti-CRD-BP antibody attached to the recombinant CRD-BP on the plate. Alternatively, a tagged secondary antibody can be used for quantitation. This secondary antibody can be tagged with an enzyme such as horseradish peroxidase or with a probe such as biotin.  
20 The amount of bound secondary antibody is then detected by standard assays and is a measure of the amount of anti-CRD-BP antibody in the serum of the patient.

          ii.   Antigen Capture Assay

Serum from the patient is attached to a solid  
25 support such as a microtiter well. Then radiolabeled, recombinant CRD-BP is added. Unbound CRD-BP is washed off of the plate, and the amount of bound antigen is measured. The radiolabeled CRD-BP could be labeled in

vivo in bacteria or yeast using 35S or could be radioiodinated *in vitro*.

D. Treatment of cancer by eliminating the CRD-BP from the cancer cells

5           The basic idea of the present invention is primarily based on two notions: that the CRD-BP stabilizes *c-myc* mRNA in cells and that the CRD-BP is expressed post-natally in tumor cells but not in normal cells. As a  
10       result, *c-myc* mRNA is overexpressed or inappropriately expressed in tumor cells. If the CRD-BP could be eliminated, then *c-myc* mRNA would be destabilized. If *c-myc* mRNA were essential for growth or viability of the tumor cells, then the tumor cells would stop growing or  
15       die. Selectivity would be assured if the CRD-BP were expressed more abundantly in tumor cells.

Two approaches are preferred for interfering with the interaction of the CRD-BP with *c-myc* mRNA:

1. Genetic engineering

20           The way we destabilized *c-myc* mRNA in our cell-free mRNA decay system was to add excess competitor RNA to the reactions. The RNA contains the 180 nucleotides of the *c-myc* mRNA coding region determinant (CRD). The competitor RNA is thought to titrate the CRD-BP from *c-myc* mRNA. As a result, the CRD of *c-myc* mRNA is not  
25       shielded by the CRD-BP, and the mRNA is rapidly degraded by a ribonuclease.

In order to exploit a similar strategy in intact cells, it would be necessary to apply the techniques of

genetic engineering to overexpress *c-myc* mRNA CRD RNA in the affected tissue or organ. One might introduce DNA capable of expressing the CRD competitor RNA in the tissue or organ. Alternatively, it might be feasible to introduce a ribonuclease-resistant, long-lasting form of CRD RNA itself. It is important to note that specificity would be achieved if the target cancer cells were expressing the CRD-BP, while non-cancer cells did not express it. Under these conditions, the competitor CRD RNA would have a deleterious effect only on the cancer cells.

2. Use of an inhibitor that blocks CRD-BP binding to the *c-myc* mRNA CRD

We presume that the CRD-BP folds in such a way that it is able to recognize a particular segment of *c-myc* mRNA, namely, the CRD RNA segment. One could design peptide or nucleic acid analogues or other compounds that bind to the CRD-BP so as to inhibit its ability to interact with *c-myc* mRNA in cells. This is similar to strategies that are being considered by pharmaceutical companies hoping to design antiviral compounds capable of entering cells and interacting with viral-derived proteins and nucleic acids. The protease inhibitors used in HIV-infected patients are an example of a pharmaceutical agent directed against a specific viral-encoded product.



## EXAMPLES

A. Experimental ProceduresCell lines and preparation of subcellular fractions.

All cell lines were obtained from the American Type  
5 Culture Collection (Rockville, MD). K562 human  
erythroleukemia cells were cultured in RPMI-1640 medium  
containing 10% calf serum plus a penicillin/streptomycin  
mix. NIH/3T3 cells were grown in DMEM (4.5 g/L glucose)  
containing 10% calf serum and antibiotics. All  
10 antibiotics and sera were from Gibco/BRL Life  
Technologies.

Subcellular fractions were prepared as follows. All  
steps following cell harvesting were at 4°C. Cells were  
grown in 1 liter spinner flasks to a density of  $3-5 \times 10^5$   
15 cells/ml. They were harvested, collected by low speed  
centrifugation, and washed 3 times with cold F12 medium  
without serum. The cell pellet was resuspended at a  
density of  $1.5 \times 10^7$  cells/ml in Buffer A (1 mM potassium  
acetate, 1.5 mM magnesium acetate, 2 mM DTT, 10 mM  
20 Tris-Cl, pH 7.4) containing 100 mM EGTA, 100 mg/ml PMSF,  
and 2 mg/ml each of aprotinin, leupeptin, and pepstatin A  
(all from Sigma). The cells were lysed with 30-40  
strokes of a Dounce homogenizer, and the lysate was  
centrifuged for 10 minutes at 20,000 x g to pellet nuclei  
25 and other organelles. The supernatant (S20) was layered  
over a cushion of 30% (w/v) sucrose dissolved in Buffer A  
and was centrifuged for 2.5 hours at 130,000 x g to  
pellet polysomes. The supernatant (S130) above the

sucrose cushion was harvested, and the polysomal pellet was resuspended in Buffer A containing PMSF, leupeptin, pepstatin A, and aprotinin. The S20 pellet (crude nuclei) was washed once in Buffer A and centrifuged, and  
5 the nuclear wash material in the supernatant was harvested and saved. The pelleted, washed nuclei were then resuspended in 300  $\mu$ l of Buffer B (1.5 mM MgCl<sub>2</sub>, 140 mM NaCl, 20% glycerol, 10 mM Tris-Cl, pH 8.0) and lysed by adding 2.7 ml of Buffer C (5.0 % SDS, 10% glycerol, 5%  
10  $\beta$ -mercaptoethanol, 62.5 mM Tris-Cl, pH 6.8). The extract was then passed 10 times through an 18-gauge needle and boiled for 15 minutes. To isolate ribosomal salt wash (RSW) from either tissue culture cells or reticulocyte translation reactions, an aliquot of polysomes was  
15 incubated for 20 minutes at 4°C with 1 M NaCl in buffer A, followed by centrifugation for 2.5 hours at 130,000 x g to re-pellet the salt washed polysomes (26). Glycerol was added to 10% to the supernatant (RSW) above the sucrose cushion, and the salt-washed polysomes were  
20 resuspended in Buffer A containing the protease inhibitors. All fractions were stored at -70°C.

Protein purification and microsequencing. The human c-myc CRD-BP was purified from K562 cell RSW as described (31). Two independent preparations of CRD-BP from  
25 different RSW isolates were microsequenced for this study. The first sequence was determined at the Protein Sequence and Peptide Synthesis Facility of the University of Wisconsin Biotechnology Center (Madison, WI). The

second sequence was distinct from the first, did not overlap, and was determined at the Keck Laboratories, Yale University (New Haven, CT). The second sequence was used for preparing PCR primers.

5    Cloning of mouse CRD-BP cDNA.

1.    CRD-BP cDNA cloning. We first prepared a human CRD-BP cDNA and used its sequence to identify mouse CRD-BP cDNA. DNA oligomers were synthesized by the Nucleic Acid Sequence and Oligomer Synthesis Facility of  
10    the University of Wisconsin Biotechnology Center (Madison, WI) or by GIBCO-BRL Life Technologies (Grand Island, NY). A K562 (human) cell cDNA lambda library (Clontech, Palo Alto, CA) was first screened by degenerate PCR in order to amplify a 45 bp DNA sequence  
15    based on the 15 amino acids of the second CRD-BP peptide sequence. The following primers were used:  
5'-GTBAAYGARYTBCARAA-3' (coding) (SEQ ID NO:31) and  
5'-GGVACVACVACYTCDGC-3' (non-coding) (SEQ ID NO:32). The conditions were 30 cycles, 94°C for 30 seconds, 45°C for  
20    30 seconds, 72°C for 1 minute, AMPLITAQ DNA Polymerase (Perkin Elmer). PCR products from this and subsequent reactions were subcloned directly into pT7-Blue (Novagen, Madison, WI) for sequencing, which was performed by PCR  
25    using the ABI Prism AmpliTaq FS Dye Terminator Reaction Kit (Applied Biosystems, Inc.) according to the manufacturer's recommendations. A 45 bp product encoding the expected 15 amino acid sequence was isolated in this way. The same cDNA library was then used for non

degenerate PCR with a CRD-BP-specific coding primer from the middle of the 45 bp sequence (5'-GCTGCCGTCAAATTCTG-3') (SEQ ID NO:33) plus a lambda-specific primer (5'-TCGACGGTTTCCATATG-3') (SEQ ID NO:34) under the following conditions: 30 cycles, 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 3 minutes, AMPLITAQ DNA Polymerase. This step generated a 227 bp cDNA. The same library was then plated, transferred in duplicate to nitrocellulose filters, and screened by hybridization with the 227 bp <sup>32</sup>P-DNA as probe. This step generated a 1069 bp partial human CRD-BP cDNA with an open reading frame encoding both of the peptides obtained by sequencing purified CRD-BP. This cDNA did not contain the 5' part of the coding region, the 5'-UTR, or most of the 3'-UTR.

To complete the cloning of the 3' terminal region, 3' rapid amplification of cDNA ends (3'-RACE) was performed. Oligomer Not(dT) (5'-AACCCGGCTCGAGCGGCCGC TTTTTTTTTTTTTTTTTT-3') (SEQ ID NO:35) and Superscript II (GIBCO-BRL) were used according to the manufacturer's recommendations to reverse transcribe 0.5 µg of K562 cell poly(A)+ mRNA. The cDNA template was then amplified using VENT DNA Polymerase (New England Biolabs) with oligomers CRD-BP1 (5' ACGGCAGCTGAGGTGGTAGTACC-3') (SEQ ID NO:36) and NotAdaptmer (5'-AACCCGGCTCGAGCGGCCGCT-3') (SEQ ID NO:37) as 5' and 3' primers, respectively. Conditions were 1 cycle of 94°C for 1 minute, followed by 35 cycles

of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1.5 minutes.

2. Cloning of mouse CRD-BP cDNA. The partial human CRD-BP cDNA generated as described above was used to identify mouse CRD-BP cDNAs in the EST Database using the NCBI Blast Program. The larger of the two EST's, AA073514, was obtained from Genome Systems, Inc. (St. Louis, MO) and was sequenced. The amino acid sequence it encoded was 99% identical to that of our human CRD-BP, indicating that it corresponded to the mouse CRD-BP. It contained the entire 3'-UTR and most of the coding region. To extend the 5' sequence, 5'- RACE was performed on a 17 day mouse embryo Marathon-Ready cDNA Library (Clontech) using ADVANTAGE KlenTaq DNA Polymerase (Clontech) according to the manufacturer's instructions. In primary reactions, "touchdown PCR" was performed with oligomers AP1 (Clontech) and CRD-BP2 (5'-AGGTTCCGTCCTTCCTTGCCAATG-3') (SEQ ID NO:38) as 5' and 3' primers, respectively. Conditions were 1 cycle of 94°C for 1 minutes, 5 cycles of 94°C for 10 seconds, 72°C for 7.5 minutes, 5 cycles of 94°C for 10 seconds, 70°C for 7.5 minutes, 20 cycles of 94°C for 10 seconds, 68°C for 7.5 minutes, 10 cycles of 94°C for 10 seconds, 60°C for 20 seconds, 68°C for 7.5 minutes. DNA bands were excised from a 1% agarose gel, and secondary PCR was performed with them using nested 5' and 3' primers [oligomers AP2 (Clontech) and CRD-BP3 (5'-AACTTCATCTGCCGTTTTGG 5') (SEQ ID NO:39), respectively]. Conditions were 1 cycle of

94°C for 1 minutes, followed by 25 cycles of 94°C for 15 second, 60°C for 30 seconds, 68°C for 5 minutes. Since the resulting clone did not contain the translation start site or any 5'-UTR, a mouse BAC library was screened for  
5 the CRD BP gene by PCR with primers CRD-BP4 (5'-CATCAACTGGAGAACCATG-3') (SEQ ID NO:40) and CRD-BP5 (5'-GACTGCGTCTGTTTGTGATG-3') (SEQ ID NO:41). A BAC clone containing the mouse CRD-BP gene was obtained from Genome Systems. The remainder of the coding region and  
10 at least part of the 5'UTR was sequenced from this BAC clone using oligomer CRD BP6 (5'-CTGTAGGAGATCTTGCTC-3') (SEQ ID NO:42) as primer. Sequence comparisons were generated using the Genetics Computer Group (GCG) Bestfit and Gap algorithms. Theoretical translations were made  
15 with the GCG Translate program.

In vitro translation of mouse CRD-BP. A portion of the mouse CRD-BP cDNA was subcloned into pSPUTK (Stratagene, La Jolla, CA) to create the translation clone pSPUTK-CRD-BP as follows: A single base mutation  
20 (underlined) was made in the 5' primer (5'CGCACCGCCACCATGGACAAGCTTTACATCGG-3') (SEQ ID NO:43) to generate an NcoI site for subcloning. The mutation changes an asparagine to an aspartic acid. The 3' primer (5'-ACTGGGATCTGACCCATCCT-3') (SEQ ID NO:44) was from the  
25 CRD-BP 3'-UTR. Conditions were 1 cycle of 94°C for 1 minute, followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 3 minutes. pSPUTK-CRD-BP, pSPUTK-Luciferase, or pSPUTK vector templates were

transcribed and translated using the TnT® Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

Immunoprecipitation, immunoblotting, and gel

- 5 retardation assays. Immunoprecipitation (IP) of 60S ribosomal subunits was performed essentially as previously described (33). Briefly, human anti-P protein serum (Immunovision) or normal human serum was conjugated to Protein G-Plus Sepharose beads (Oncogene Science).
- 10 The anti-P protein serum recognizes three large ribosomal subunit proteins ( $P_0$ -38 kDa,  $P_1$ -19 kDa,  $P_2$ -17 kDa; ref. 34). K562 polysomes were dissociated into mRNP and ribosomal subunits by incubation with 20 mM EDTA at 4°C for 20 minutes. Protein G-Plus Sepharose-conjugated
- 15 antibodies were then incubated with 10  $\mu$ l of the dissociated polysomes in IP buffer (100 mM KCl, 5 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 100  $\mu$ g/ml PMSF, 0.5% aprotinin, and 2  $\mu$ g/ml each leupeptin and pepstatin A, 10 mM HEPES, pH 7.3) for 16 hours at 4°C with gentle mixing.
- 20 The beads were washed three times for 20 minutes each at 4°C in IP buffer. Bound proteins were eluted by resuspending the beads in Buffer D (2.3% SDS, 10% glycerol, 62.5 mM Tris-Cl, pH 6.8) and incubating the beads at 95°C for 5 minutes.
- 25 Immunoblotting was performed as previously described (32). For CRD-BP, the primary antibody was a chicken anti-CRD-BP IgY raised against the purified human protein (31, 32), and the secondary detection antibody was

horseradish peroxidase (HRP) conjugated rabbit anti-chicken IgY (Promega). For the ribosomal P proteins, human anti-P protein serum (see above) was the primary antibody, and the secondary detection antibody was HRP-conjugated goat anti-human IgG (Promega). For heat shock protein-90 (HSP 90), the primary antibody was a rabbit anti-mouse HSP-90 polyclonal IgG (a kind gift from Dr. Alan Poland), and the secondary detection antibody was HRP-conjugated goat anti-rabbit IgG (Sigma). Blots were developed by enhanced chemiluminescence (ECL) using either standard (Amersham) or Supersignal ULTRA (Pierce) reagents. Distinct bands were not detected with preimmune antibodies, normal human serum, or secondary antibodies alone (data not shown). Where noted, blots were stripped for 30 minutes at 50°C in 2% SDS, 100 mM  $\beta$ -mercaptoethanol, 50 mM K<sub>2</sub>HPO<sub>4</sub>, pH 6.8 and were then washed extensively in buffer containing 5% nonfat dry milk to remove SDS and  $\beta$ -mercaptoethanol. Gel retardation assays were performed as previously described (31, 32).

Sucrose gradient centrifugation and ribosomal RNA analysis. All procedures were performed at 4°C. For analyzing the CRD-BP association with ribosomal subunits, an aliquot of K562 cell polysomes (50  $\mu$ l) or cytoplasmic lysate (S20; 150  $\mu$ l) was brought to a final concentration of 20 mM EDTA. The material was mixed gently, left on ice for 20 minutes, layered over a 10 ml linear 5-30% sucrose gradient in Buffer E (100 mM KCl, 10 mM potassium



acetate, 5 mM EDTA, 1 mM DTT, 5 mM HEPES, pH 7.3) (33), and centrifuged in a Beckman SW41.1 rotor for 4 hours at 4°C, 38,000 rpm (178,000 x g). Following centrifugation, 500 µl fractions were pipetted sequentially from the top of the gradient. The pellet at the bottom of the tube was resuspended in 500 µl of Buffer E containing 5% sucrose. Proteins were precipitated with methanol and chloroform prior to immunoblotting. RNA from each fraction was isolated using TRIzol reagent (Gibco/BRL) following the manufacturer's directions and was electrophoresed in a 1% agarose gel containing 10 mM sodium acetate, 1 mM EDTA, 40 mM MOPS, pH 7.0. Ribosomal RNA bands were visualized by staining with ethidium bromide (0.05 µg/ml).

Recombinant, 35S-labeled CRD-BP or luciferase was synthesized in reticulocyte extracts and analyzed by sucrose gradient centrifugation essentially as previously described (35) with slight modifications. The reactions (100 µl) were chilled on ice, layered over a 4 ml linear 20-40% sucrose gradient containing 25 mM potassium acetate, 1.5 mM magnesium acetate, 1 mM DTT, 20 mM Tris-Cl, pH 7.2, and centrifuged in a Beckman SW60 rotor for 5 hours at 4°C, 133,000 x g. Fractions were pipetted sequentially from the top of the gradient, and 5 µl of each were electrophoresed in a 10% SDS-PAG. Full length CRD-BP and luciferase protein were quantified by PhosphorImager analysis using the ImageQuant program (Molecular Dynamics). Ribosomal RNA from each fraction

was extracted, electrophoresed in a 1% agarose gel, and visualized by staining with ethidium bromide.

## B. Results

### Cloning the cDNA encoding the CRD-BP, a novel

5 KH-domain RNA binding protein. Two preparations of highly purified CRD-BP were isolated from human K562 cell polysomes in separate experiments. Each preparation was microsequenced, and each gave a different, nonoverlapping sequence, which was P-A-Q-V-G-A-I-Q/I-G-k/r-I/K-Y/G-  
10 Q-X-i/l-k (SEQ ID NO:45) from the first and -N-E-L-Q-N-L-T-A-A-E-V-V-V-P (SEQ ID NO:46) from the second. Lower case letters indicate residues of less confidence than upper case letters. A K562 cDNA library was then screened by PCR using degenerate primers based  
15 on the amino and carboxy termini of the second peptide (Experimental Procedures). A 45 bp product was generated, subcloned, sequenced, and found to encode the second amino acid sequence. Subsequent PCR amplification and library screening identified a 1069 bp partial human  
20 cDNA containing an open reading frame (ORF) that included both peptide sequences obtained by microsequencing.

In order to continue our analysis of the properties and developmental regulation of the mouse CRD-BP, we then exploited the human cDNA sequence to isolate a putative  
25 mouse CRD-BP cDNA (Experimental Procedures). A clone containing at least a portion of the 5'-UTR, a complete coding region, and a complete 3'-UTR was obtained and sequenced (Fig.1). Two in-frame AUG start codons are

present near the 5' terminus of the cDNA. We have tentatively designated the downstream AUG as the translation start site, because it is embedded within a sequence that is preferred as a translation start signal (36). In contrast, the upstream AUG is not within a preferred translation start motif.

The predicted sequence of the murine cDNA contains several KH domains and an RGG box, which are characteristic motifs found in some RNA-binding proteins. There are four KH domains arranged as two pairs of repeats (Fig. 1, double underlines). Each repeat pair is separated by approximately 30 residues, and the two pairs of repeats are separated by 78 residues. The putative RGG box (boxed) is located upstream of the KH domains. There are two putative nuclear export signals (overlined). One is similar to that found in the FMR RNA-binding protein (FMRP), which is associated with familial mental retardation (37-39). The other is similar to that in the HIV Rev protein. There is also a putative nuclear localization signal (underlined).

The RGG, nuclear export, and KH domain regions of the CRD-BP are similar to those found in several other RNA-binding proteins (Fig. 2). Moreover, the human and murine CRD-BP sequences are similar to a human cDNA called hKOC, an acronym for human KH domain protein overexpressed in human cancer (Fig. 2). The hKOC open reading frame encodes a protein of unknown function that was cloned on the basis of its overexpression in human

pancreatic cancer tissue (40). The mouse CRD-BP coding region is 88.8% and 99.1% identical to the coding region of the human CRD-BP at the nucleic acid and protein sequence levels, respectively. For comparison, mouse  
5 CRD-BP is 66.6% and 74.0% identical to the hKOC coding region at the nucleic acid and protein levels, respectively. Based on these comparisons and on the data presented below, we conclude that our cDNA encodes CRD-BP and is not the mouse homologue of human KOC. Additional  
10 evidence (presented below) suggests that the CRD-BP and hKOC are members of a new subfamily of KH domain containing RNA-binding proteins.

Comparison of *in vitro* synthesized CRD-BP with cell-derived CRD BP. To determine whether our murine  
15 cDNA clone encoded full-length CRD-BP with the expected properties of a *c-myc* mRNA-binding protein, we synthesized the protein *in vitro* and analyzed it by immunoblotting and gel retardation assays. Reticulocyte transcription/translation reactions were programmed with  
20 CRD-BP cDNA subcloned into a pSPUTK vector. The CRD-BP sequences in the subclone began with the AUG denoted as the translation start site in Fig. 1. This subclone did not contain the upstream, in-frame AUG. The translation extract was fractionated by SDS-PAGE and analyzed by  
25 immunoblotting with anti-CRD-BP antibody. A protein of ~68 kDa from the cDNA translation was recognized by anti-CRD-BP antibody and migrated close to the positions of authentic CRD-BP from human (K562) and mouse (NIH/3T3)

cells (Fig. 3, lanes 1-3). An immunoreactive band was not observed in control lanes containing extract programmed with the pSPUTK vector (Fig. 3, lane 4) or with luciferase cDNA (data not shown), indicating that the antibody specifically detected CRD-BP and not an endogenous reticulocyte protein. Therefore, our cDNA encodes CRD-BP. The cross-reacting band (p85) seen in the K562 and NIH/3T3 RSW lanes is a protein observed previously (32). Its identity and function are unknown. p85 does not bind *c-myc* CRD RNA (32), and it localizes to different subcellular fractions when compared to CRD-BP (see below).

Gel retardation assays were performed to determine if recombinant CRD-BP could bind specifically to *c-myc* CRD RNA. In preliminary experiments, we noted that most of the recombinant CRD-BP co-fractionated with reticulocyte ribosomes (see below). Therefore, the gel retardation assays were performed using RSW from cells or from reticulocyte translation reactions. RSW's were incubated with *c-myc* CRD <sup>32</sup>P-RNA, and RNA/protein complexes were resolved from free <sup>32</sup>P-RNA by non-denaturing gel electrophoresis. An RNA/protein complex was observed with protein from K562 cells and from the translation extract programmed with CRD-BP cDNA (Fig. 4A, lanes 1 and 2, respectively). These complexes migrated to similar or identical positions in the gel. An RNA/protein complex was not observed with protein from the luciferase (Luc), Vector, or no mRNA (None) control

reactions (Fig. 4A, lanes 3-5). Therefore, *in vitro* synthesized CRD-BP, like its cell-derived counterpart, associates with *c-myc* CRD RNA *in vitro*.

Previous work had shown that cell-derived CRD-BP did  
5 not bind to other RNAs we tested, suggesting that it had considerable specificity for *c-myc* CRD RNA (30, 31). A competition assay was performed to determine if . recombinant CRD-BP exhibited similar specificity. RNA-protein binding reactions contained *c-myc* CRD <sup>32</sup>P-RNA  
10 as probe plus RSW as a protein source. Reactions were supplemented with no competitor RNA or with a 200-fold molar excess of either unlabeled *c-myc* CRD RNA or  $\beta$ -globin RNA. The CRD BP/CRD <sup>32</sup>P-RNA complex was competed by excess unlabeled CRD RNA but not by  $\beta$ -globin RNA (Fig.  
15 4B). This result further confirms that this cDNA encodes functional *c-myc* CRD-BP.

Co-fractionation of recombinant CRD-BP with ribosomes in reticulocyte extracts. As noted above, preliminary experiments had indicated that a large  
20 percentage of recombinant CRD-BP co-sedimented with reticulocyte polysomes. It was important to confirm this finding, because reticulocytes contain no *c-myc* mRNA as measured by Northern blotting. Therefore, it was possible that the CRD-BP, like the FMRP (33), has an  
25 affinity for ribosomes even in the absence of what we believe to be its natural mRNA ligand. 35S-Labeled CRD-BP and luciferase were synthesized in reticulocyte extracts, and each extract was sedimented in a sucrose

gradient. Fractions were collected and assayed for ribosome content by gel electrophoresis and for protein by gel electrophoresis and PhosphorImager analysis.

Whereas all of the luciferase sedimented near the top of the gradient (Fig. 5, unfilled circles), greater than 95% of the CRD-BP co sedimented with monosomes and ribosomal subunits (filled circles). Therefore, the CRD BP can bind *in vitro* to ribosomes and ribosomal subunits in the absence of *c-myc* mRNA.

10        Localization of CRD-BP to the cytoplasm and co-fractionation with ribosomes and ribosomal subunits.

The CRD-BP is located primarily in the cytoplasmic fraction of K562 cell extracts, and much of it is associated with polysomes (ref. 31 and data not shown). This observation is consistent with its putative role as an mRNA-binding protein. However, the amount of CRD-BP per K562 cell exceeds the amount of *c-myc* mRNA by at least 1000-fold (31). Several factors could account for the "excess" CRD-BP in these cells: i) The CRD-BP might be associated with other mRNAs besides *c-myc*. ii) A portion of it might associate with ribosomes and/or ribosomal subunits, as is the case with FMRP (33). An association between the CRD-BP and ribosomes in cells would be consistent with the association of newly synthesized CRD-BP with reticulocyte ribosomes (Fig. 5). Experiments are in progress to determine whether the CRD-BP is bound to *c-myc* mRNA in cells. To determine how much of it co-fractionates with cell ribosomes and

ribosomal subunits and how much, if any, co-fractionates with nuclei, exponentially growing K562 cells were harvested, lysed, and separated into 6 fractions (Experimental Procedures). Equal cell equivalents of each fraction were analyzed by immunoblotting with an anti-CRD-BP antibody. At least 95% of the total cell CRD-BP was in the polysome fraction, and greater than 90% of this CRD-BP was eluted in the one molar salt wash (Fig. 6A, RSW). Little or no CRD-BP was detected in fractions containing nuclei or post-polysomal supernatant (Fig. 6A, Nuclei and S130, respectively). The absence of CRD-BP in these fractions could not be explained by indiscriminate proteolysis during sample preparation, because HSP-90 was detected in all of the fractions (Fig. 6B). Some p85 was detected in both the nuclear and polysomal fractions. This result, coupled with those presented below, further confirms that the CRD-BP and the cross-reacting p85 do not co-localize in cells and are functionally distinct proteins.

To determine if at least some CRD-BP is associated with ribosomal subunits, K562 cell polysomes were purified by centrifugation and then resuspended in a buffer containing 20 mM EDTA, which dissociates polysomes into ribosomal subunits and free mRNP. The EDTA-treated polysomes were then fractionated in a sucrose gradient. Each gradient fraction plus material in the pellet at the bottom of the tube were analyzed for ribosomal RNA content by gel electrophoresis and for CRD-BP by



immunoblotting. The small ribosomal subunits sedimented primarily in fractions 6-11, while the large subunits were in fractions 10-14 (Fig. 7, panels A and B). The CRD-BP co-sedimented with the subunits and was also  
5 detected in the pelleted material, which is expected to contain undissociated polysomes and monosomes (Fig. 7C). Therefore, the CRD-BP co-fractionates with ribosomal subunits in K562 cells. The nature of the CRD-BP/subunit association is unclear. In view of the broad  
10 fractionation range of the CRD-BP, we have not attempted to quantitate relative CRD-BP levels from one fraction to the next.

Data from gel retardation and RNA-protein binding experiments indicate that p85 does not bind to the *c-myc*  
15 CRD RNA (31, 32). Fig. 7C also shows that the small portion of p85 that does co-pellet with polysomes is not bound to the dissociated ribosomal subunits. Rather, it sediments at the top of the gradient (Fig. 7C). Similar results were obtained using crude cytoplasmic lysate  
20 (S20) treated with EDTA (data not shown). In summary, p85 reacts with polyclonal anti-CRD-BP antibody but does not bind to *c-myc* CRD RNA (30, 31) and does not co-fractionate with the CRD-BP in cell lysates.

To verify the association of the CRD-BP with  
25 ribosomal subunits using an independent method, immunoprecipitation (IP) experiments were performed using P protein antibodies, which react specifically with proteins associated with the large (60S) subunit. K562

cell polysomes were dissociated into subunits in the presence of 20 mM EDTA and IP'd with anti-P antibody serum or normal human serum. The IP'd proteins were then analyzed by immunoblotting using antibodies against the P-proteins and the CRD-BP. The anti-P protein antibodies IP'd the three 60S proteins ( $P_0$ ,  $P_1$ , and  $P_2$ ), as expected (Fig. 8A, lane I). None of these proteins were IP'd by normal human serum (lane N). The anti-P protein antibodies also IP'd the CRD-BP (Fig 8B, lane I). These findings confirm that the CRD-BP is associated with ribosomal subunits in K562 cell extracts.

### C. Discussion

The CRD-BP is thought to stabilize *c-myc* mRNA by shielding its coding region from endonucleolytic attack (22, 30, 31). In this respect, it might be similar to the iron response protein that binds to and protects the 3'-UTR of transferrin receptor mRNA (reviewed in 41). However, the CRD-BP differs from the iron response protein and from many other mRNA-binding proteins in at least two ways. (i) Most such proteins bind within the 3'-UTR, while the CRD-BP binds to the *c-myc* mRNA coding region. It does not bind *in vitro* to RNA substrates from either of the *c-myc* untranslated regions (30). The coding region of *c-fos* mRNA also contains an mRNA half-life determinant that is a protein-binding site (42). Perhaps the function of the *myc* and *fos* mRNA coding region determinants and their respective binding proteins is related to the regulation of *myc* and *fos*

protein expression. (ii) The *c-myc* CRD-BP is developmentally regulated, being expressed abundantly in fetal and neonatal life but not in adult animals (32). Perhaps the CRD-BP has a special role in embryonic/fetal  
5 development.

The CRD-BP contains four KH domains and an RGG box, and it co-fractionates with polysomes and ribosomal subunits. These findings are consistent with it being an RNA-binding protein whose function is related in some way  
10 to translation and/or mRNA metabolism. The CRD-BP also co-fractionates with ribosomes in the absence of *c-myc* mRNA (Fig. 5). Perhaps it is bound both to *c-myc* mRNA and to ribosomes in intact cells. If so, it might be carried along with the translating ribosomes as a  
15 reservoir to be used when needed to bind to any unprotected *c-myc* mRNA molecules. The CRD-BP also contains a putative nuclear localization sequence and two putative nuclear export sequences (Figs. 1 and 2). We do not know if the CRD-BP shuttles between the nucleus and  
20 the cytoplasm. If it does shuttle, however, it appears to spend most of its time in the cytoplasm of growing cells, because little of it is detected in the nucleus at steady-state (Fig. 6).

Consistent with the unique features of the CRD-BP  
25 noted above, the CRD-BP and hKOC protein appear to represent a unique subfamily of KH domain-containing RNA binding proteins. Other putative RNA-binding proteins, including the FUSE-binding protein, P-element somatic

inhibitor, and *C. elegans* M88.5, resemble the CRD-BP in containing four KH domains (43). However, several structural features of these proteins distinguish them from CRD-BP and hKOC. The KH domains of the P-element somatic inhibitor and FUSE-binding proteins are located toward their amino termini and are organized as an evenly-spaced, four unit repeat. These proteins also contain either glycine rich or glutamine-rich stretches in their amino and carboxy termini. The overall organization of the four KH domains of M88.5 is most similar to CRD-BP and hKOC. It contains two pairs of KH domains separated by 83 amino acids. However, in contrast to CRD-BP and hKOC, the amino terminus of M88.5 is glutamine-rich and lacks an RGG box. The FUSE-binding protein contains a sequence resembling an RGG box, but this sequence is located between the third and fourth KH domains, which is not the case for the CRD-BP and hKOC protein. Finally, the core sequences of the KH domains of these other proteins are very different from those in either CRD-BP or hKOC.

Several structural and functional similarities are also noted between the CRD-BP and the FMRP, the protein encoded by the FMR1 gene, mutations in which are responsible for the most common form of inherited mental retardation (44, 45). Both proteins contain KH domains and an RGG box (37, 38) as well as nuclear import and export signals (39). Both proteins associate with ribosomes and probably with mRNA as well (33, 49, 46).

Neither protein is required for cell viability, because individuals who fail to express FMRP survive, while perfectly normal adult animals do not express the CRD-BP at levels detectable by immunoblotting and/or gel retardation assays (32). There are also some significant differences between FMRP and CRD-BP, particularly in their expression patterns. Both are expressed abundantly during fetal life, but only FMRP is detected in adult tissues (47-49).

10       The structural features of the CRD-BP and its developmental regulation pattern suggest that it might be an oncofetal protein, for the following reasons: (i) It is expressed abundantly only in fetal and neonatal life (32). (ii) All of the mouse CRD-BP EST's that are  
15       currently in the database are derived from either fetal tissue or from cell lines, including embryonic stem cells. These include AA073173 (from 13 day old embryonic heart tissue), AA619650 and AA399833 (from a pre-implantation blastocyst), AA073514 (from the P19  
20       embryonic carcinoma cell line treated with retinoic acid), and D76662 and D76781 (from the F9 embryonic carcinoma cell line). (iii) The CRD-BP is expressed in many cell lines, all of which are neoplastic or pre-neoplastic. It is expressed at high levels in K562,  
25       HeLa, and 3T3 cells (Figs. 3 and 4 and data not shown) and at low levels in other lines such as HL60, a human promyelocytic leukemia cell, and H4IIE, a rat hepatoma cell (data not shown). (iv) It is similar but not

identical to the hKOC protein that is overexpressed in pancreatic cancer and in some other tumors (Fig. 2 and ref. 40). If the CRD-BP is an oncofetal protein, it would join a growing list of RNA-binding proteins that influence the early development of the organism and/or that affect carcinogenesis. For example, mutations in the Elav proteins influence *Drosophila* development (reviewed in 50-53), while mutations in other RNA-binding protein genes result in male infertility or mental retardation (44).

D. Detecting the CRD-BP in Clinical Samples

Human tumor tissues were provided by physicians and surgeons at the UW-Madison Clinical Cancer Center. The tissues were homogenized, and a crude cytoplasmic extract was prepared. The extract was then fractionated by two-dimensional gel electrophoresis at Kendrick Laboratories (Madison, WI). Following electrophoresis in the second dimension, the proteins were transferred to PVDF membranes and returned to our laboratory.

CRD-BP was visualized by incubating the membranes with antibodies to mouse CRD-BP. These antibodies cross-react with human CRD-BP.

Findings are as follows:

1. We detect abundant CRD-BP in human breast cancer, colon cancer, and pancreatic cancer tissues. We expect to find similar results with other non-hemopoietic cancers.

2. A significantly smaller amount of CRD-BP is detected in one normal human breast tissue sample.

3. No CRD-BP is detected in several human leukemia samples.

5 Our conclusion from these studies is that the CRD-BP is overexpressed in non-leukemia human carcinomas.

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## CLAIMS

I claim:

1. A method of diagnosing the presence or absence of cancer in a human patient comprising the steps of:

a) examining patient tissue for the CRD-BP expression level; and

5 b) comparing the result of step (a) with the expression level in non-cancerous tissue from the same source, wherein an increased CRD-BP level in the patient tissue compared to the non-cancerous tissue is diagnostic of cancer.

2. The method of claim 1 wherein the detection of CRD-BP comprises the step of homogenizing biopsy tissue and obtaining a crude protein extract and examining that extract for the CRD-BP level.

3. The method of claim 2 wherein the detection is via a two antibody sandwich assay.

4. The method of claim 2 wherein the detection is via antigen competition assay.

5. The method of claim 3 wherein the detection is via antibody capture assay.

6. The method of claim 2 wherein the detection of CRD-BP is via immunoblotting.

7. The method of claim 1 wherein the detection of CRD-BP takes place in cells via immunological or *in situ* hybridization methods.

8. The method of claim 1 wherein the cancer is selected from the group consisting of breast cancer, colon cancer and pancreatic cancer.

9. The method of claim 1 wherein the patient tissue is breast tissue.

10. The method of claim 9 wherein the non-cancerous tissue is breast tissue.

11. The method of claim 1 wherein the patient tissue is colon tissue.

12. The method of claim 11 wherein the non-cancerous tissue is colon tissue.

13. The method of claim 1 wherein the tissue is pancreatic tissue.

14. The method of claim 13 wherein the non-cancerous tissue is pancreatic tissue.

15. A method of determining the stage of cancer in a human patient comprising the step of:

- a) examining patient tissues for the CRD-BP expression levels, and
- 5 b) correlating that expression level with disease prognosis.

16. A method of determining the presence or absence of anti-CRD-BP antibody in a patient's serum comprising the step of:

- a) exposing a patient's serum to CRD-BP and
- 5 determining whether an anti-CRD-BP antibody is present.

17. A method of determining the presence or absence of CRD-BP itself in a patient's serum comprising the step of:

- a) exposing a patient's serum to CRD-BP
- 5 antibody and determining whether the CRD-BP is present.

18. A method of inhibiting cancer cell growth comprising the step of eliminating or lowering the level of CRD-BP in the cancerous cells.

19. The method of claim 18 wherein ability of the CRD-BP to protect *c-myc* mRNA from rapid destruction is by providing the cell with a competitor RNA.

20. The method of claim 18 wherein the ability of the CRD-BP to protect c-myc mRNA from rapid destruction is reduced or eliminated via the use of an inhibitor that blocks CRD-BP binding to the *c-myc* mRNA CRD.

## FIG. 1A

1 GGTGGGGTGGTAGAAAGTTTGGGGCTCCCGCCCGCGTATCCACGCCCTATCGGCATAG  
 61 GAGGATATCCGGCCCGCCCGCGGATCGGCATTGAATGGAACAGTGTCTTGCCCCGC  
 121 CACCGCCACCATGAACAAGCTTTACATCGGCAACCTCAACGAGAGTGTGACCCCCGCAGA  
 1 M N K L Y I G N L N E S V T P A D  
 181 CTTGGAGAAAGTATTTCGGGAGCACAAAGATCTCCTACAGCGGCCAGTTCTTGGTCAAATC  
 18 L E K V F A E H K I S Y S G Q F L V K S  
 241 CGGCTACGCCCTTCGTGGATTGCCCCGACGAGCACTGGGCGATGAAGGCCATCGAACTTT  
 38 G Y A F V D C P D E H W A M K A I E T F  
 301 CTCGGGAAAGTAGAACTGCAAGGAAACGCTAGAGATTGAACACTCAGTCCCCAAAAA  
 58 S G K V E L Q G K R L E I E H S V P K K  
 361 ACAAAGGAGTCGGAAAAATACAGATCCGCAATATTCACCTCAGCTCCGATGGGAAGTGCT  
 78 Q R S R K I Q I R N I P P Q L R W E V L  
 421 AGATAGCCTGGCTCAGTACGGTACAGTGGAGAACTGTGAGCAAGTGAACACTGAAAG  
 98 D S L L A Q Y G T V E N C E Q V N T E S  
 481 TGAGACAGCGGTGGTCAACGTCACCTACTCTAACCGGGAGCAGACCAGGCAAGCTATCAT  
 118 E T A V V N V T Y S N R E Q T R Q A I M  
 541 GAAGCTAAATGGCCATCAACTGGAGAACCATGCCCTGAAGTCTCCTACATACCTGATGA  
 138 K L N G H Q L E N H A L K V S Y I P D E

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## FIG. 1B

601 GCAGATAACACAAGTCTGAGATGGGCGTCGTGGAGGCTTTGGGTCTCGGGGCCAGCC  
 158 Q I T Q G P E N P R R G G F G S R G Q P  
  
 661 CCGCAAGGTCGCCCGTGGCAGCAGGGGCTCCAGCCAAAGCAGCAGCCAGTGGACATCCC  
 178 R Q G S P V A A G A P A K Q Q P V D I P  
  
 721 TCTCCGGCTCCTGGTGCCCTACGCAGTATGTAGGCGCTATCATTTGGCAAGGAGGTGCCAC  
 198 L R L L V P T Q Y V G A I I G K E G A T  
  
 781 CATCCGAAACATCACAAACAGACGCAGTCCAAATAAGACGTGCATAGGAAGAGAATGC  
 218 I R N I T K Q T Q S K I D V H R K E N A  
  
 841 GGGCGCTGGGAGAAGGCCATCAGCGTGCATTCAACCCCTGAAGGCTGCTCCTCCGCGTG  
 238 G A A E K A I S V H S T P E G C S S A C  
  
 901 CAAGATGATCTTGGAGATTATGCACAAGGAGGCAAGGACACCAAAACGGCAGATGAAGT  
 258 K M I L E I M H K E A K D T K T A D E V  
  
 961 TCCCCTGAAGATCCTGGCTCATAACAACCTTCGTGGGGCGGACTCATTTGGCAAGGAAGGCCG  
 278 P L K I L A H N N F V G R L I G K E G R  
  
 021 GAACCTGAAGAAGGTGGAGCAGGACACAGAGACGAAGATCACCATCTCATCTCGCTCCAGGA  
 298 N L K K V E Q D T E T K I T I S S L Q D

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## FIG. 1C

1081 CCTCAGCTCTATAACCCCTGAGAGGACCATCACTGTGAAGGCGCCATTGAGAACTGTTG  
318 L T L Y N P E R T I T V K G A I E N C C

1141 CAGGGCCGAGCAGGAGATCATGAAGAAAGTTCGAGAGGCTTACGAGAACGACGTGGCCCGC  
338 R A E Q E I M K K V R E A Y E N D V A A

1201 CATGAGCTTGCAGTCCACCTCATCCCTGGGCTTAACCTGGCTGCTGTAGTCTCTTCCC  
358 M S L Q S H L I P G L N L A A V G L F P

1261 AGCTTCATCCAGCGTGTCCCTCCTCCAGCAGTGTCACTGGGGCTGCTCCCTATAG  
378 A S S S A V P P P S S V T G A A P Y S

1321 CTCCTTCATGCAGGCTCCGGAGCAGGAGATGGTACAAGTGTTCATCCCGCCAGGCTGT  
398 S F M Q A P E Q E M V Q V F I P A Q A V

1381 GGGCGCCATCATTTGCAAGAAGGCGCAGCACATCAAACTCTCCCGTTTCGCCAGCGC  
418 G A I I G K K G Q H I K Q L S R F A S A

1441 CTCCATCAAGATTGCTCCACCAGAAACACCTGACTCCAAAGTTCGAATGGTCTCATCAC  
438 S I K I A P P E T P D S K V R M V V I T

1501 TGGACCCCCAGGCTCAGTTCAGGCTCAGGGAAGAATTATGGCAAACTAAAAGAAGA  
458 G P P E A Q F K A Q G R I Y G K L K E E

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## FIG. 1D

1561 GAATTTCTTTGGTCCCAAGGAGGAAGTAAAGCTAGAGACCCACATACGGGTTCCGGCTTC  
478 N F F G P K E E V K L E T H I R V P A S  
1621 AGCAGCCGGCCGTCATCGGCAAGCGGCAAAACGGTGAATGAGCTGCAGAACTTGAC  
498 A A G R V I G K G G K T V N E L Q N L T  
1681 TGCAGCTGAGGTGAGTGCCAAAGAGACCAGACCCCGGATGAGAACGACCAAGTCATTGT  
518 A A E V V P R D Q T P D E N D Q V I V  
1741 TAAGATCATCGGACATTTCTATGCCAGCCAGATGGCTCAGCGGAAGATCCGAGACATCCT  
538 K I I G H F Y A S Q M A Q R K I R D I L  
1801 GGCTCAAGTTAAGCAACAGCACCAAGAGGACAGAGCAACCTGGCCACGACGGAGGAA  
558 A Q V K Q Q H Q K G Q S N L A Q A R R K  
1861 GTGACCCCGCCCTCCTGTCCCATTTGGCTCCAAGATCAGCAGGAGGAACACAGAACTGG  
578 \*  
1921 AGGGCGGGTGGAGGGCCGGTGTGTTTTTCCAGCAGGCCCTGAGAATGAGTGGGAATCAG  
1981 GGCAATTTGGCCCTGGCTGGAGATCAGGTTTGCACACTGTATTGAGAACAAATGTTCCAGTG  
2041 AGGAATCCTGATCTCTCGCCCCCAATTGAGCCAGCTGGCCACAGCCCCCCTTGGAATA  
2101 TCACCATTGCAATCATAGCTTGGGTGCTTTTAAACGTGGATTGTCTTGAAGTTCTCCAG  
2161 CCTCCATGGAAGGATGGGTCAGATCCCAGTCCAGTGGGGAAGAGAAATAAAATTTCCCTTCAGGTT  
2221 TTAT

FIG. 2A

	SEQ ID NO							
mCRD-BP	R	R	G	G	F	G	S	3
hKOC	G	R	R	G	L	G	Q	4
hnRNPK	G	R	G	G	F	-	D	5
Fibrillarin	G	R	G	G	F	G	D	6
Nucleolin	G	R	G	G	F	G	G	7
FMRP	L	R	R	G	D	G	R	8
Consensus:	G	R	G	G	F	G	R	9
								Q

FIG. 2B

	SEQ ID NO							
mCRD-BP	Q	L	R	-	W	E	V	10
hKOC	H	L	Q	-	W	E	V	11
FMRP	Q	L	R	-	L	E	R	12
mCRD-BP	T	I	S	S	L	Q	D	13
hKOC	T	I	S	S	L	Q	E	14
REV	Q	L	P	P	L	E	R	15
Consensus:	Q	L			L	E		16
	T	I			W	Q		I



**FIG. 2C-2**

FROM  
FIG. 2C-1

SEQ ID NO	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Q	V	V	I	I	I	V	I	I	T	I	I			I
D	S	T	V	K	T	T	I	K	E	L	T			I
F	I	I	V	V	I	I	V	V	I	V	I			I
L	A	T	M	I	S	T	M	V	A	V	I			I
T	K	R	R	V	K	R	R	V	A	R	R			R
S	E	E	V	Q	E	E	V	Q	S	D	D			K
A	A	P	K	V	A	P	K	V	I	S	D			
Q	A	N	S	D	A	P	K	V	D	S	E			
T	A	G	D	E	A	P	K	V	E	S	E			
E	L	Y	D	E	A	P	K	V	N	S	E			
N	L	P	D	E	A	P	K	V	L	H	S			
E	T	P	D	E	A	P	K	V	P	E	G			
K	P	P	Q	K	E	A	P	K	C	C	P			
R	Q	P	D	R	Q	E	A	P	E	E	E			
H	L	A	R	H	L	Q	E	A	P	E	E			
V	S	I	P	V	P	L	Q	E	P	R	S			
D	S	K	V	D	S	P	L	Q	P	S	S			
I	I	I	V	I	I	I	K	V	P	L	F			
-	-	-	-	-	-	-	-	-	P	I	I			
-	-	-	-	-	-	-	-	-	S	K	I			
-	-	-	-	-	-	-	-	-	V	-	-			
K	K	A	E	K	K	A	S	E	S	T	A			
S	T	A	A	S	T	A	S	A	A	S	I			
Q	E	A	A	Q	D	A	S	A	N	Q	I			
T	A	A	Q	T	T	A	S	A	T	G	D			
D	F	L	Q	D	F	L	S	S	E	P	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N	G	L			
L	Q	D	T	T	L	S	S	S	N	G	L			
D	F	L	Q	D	F	L	S	S	N</					

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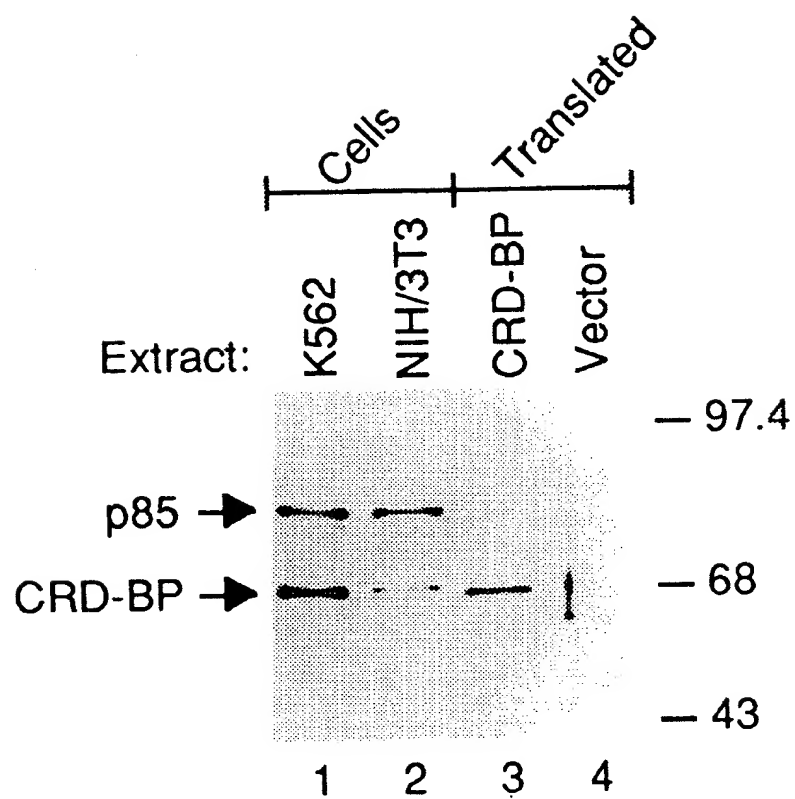


FIG. 3

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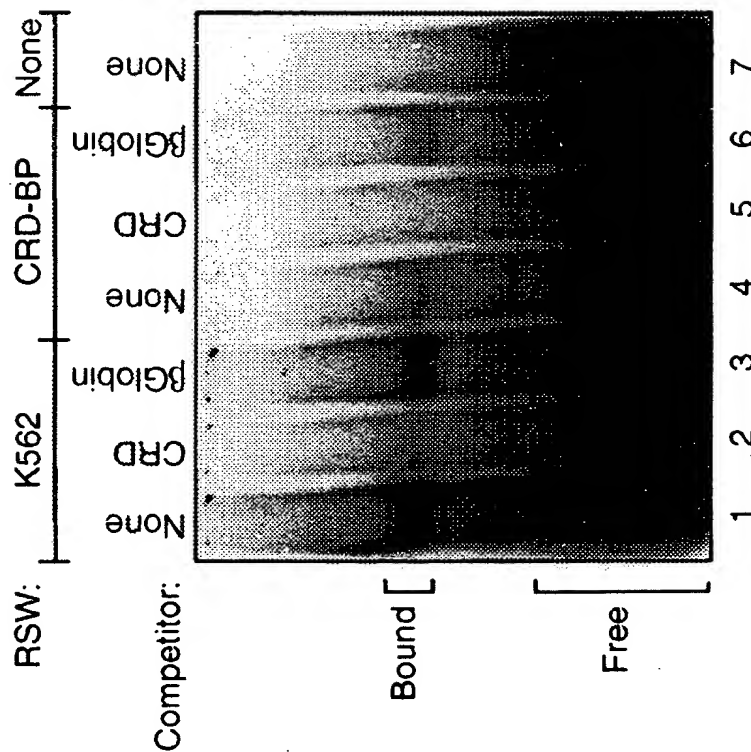


FIG. 4B

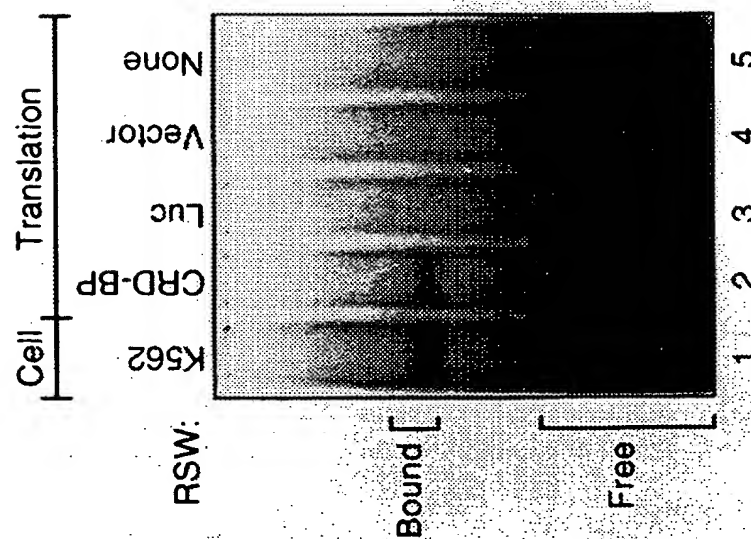
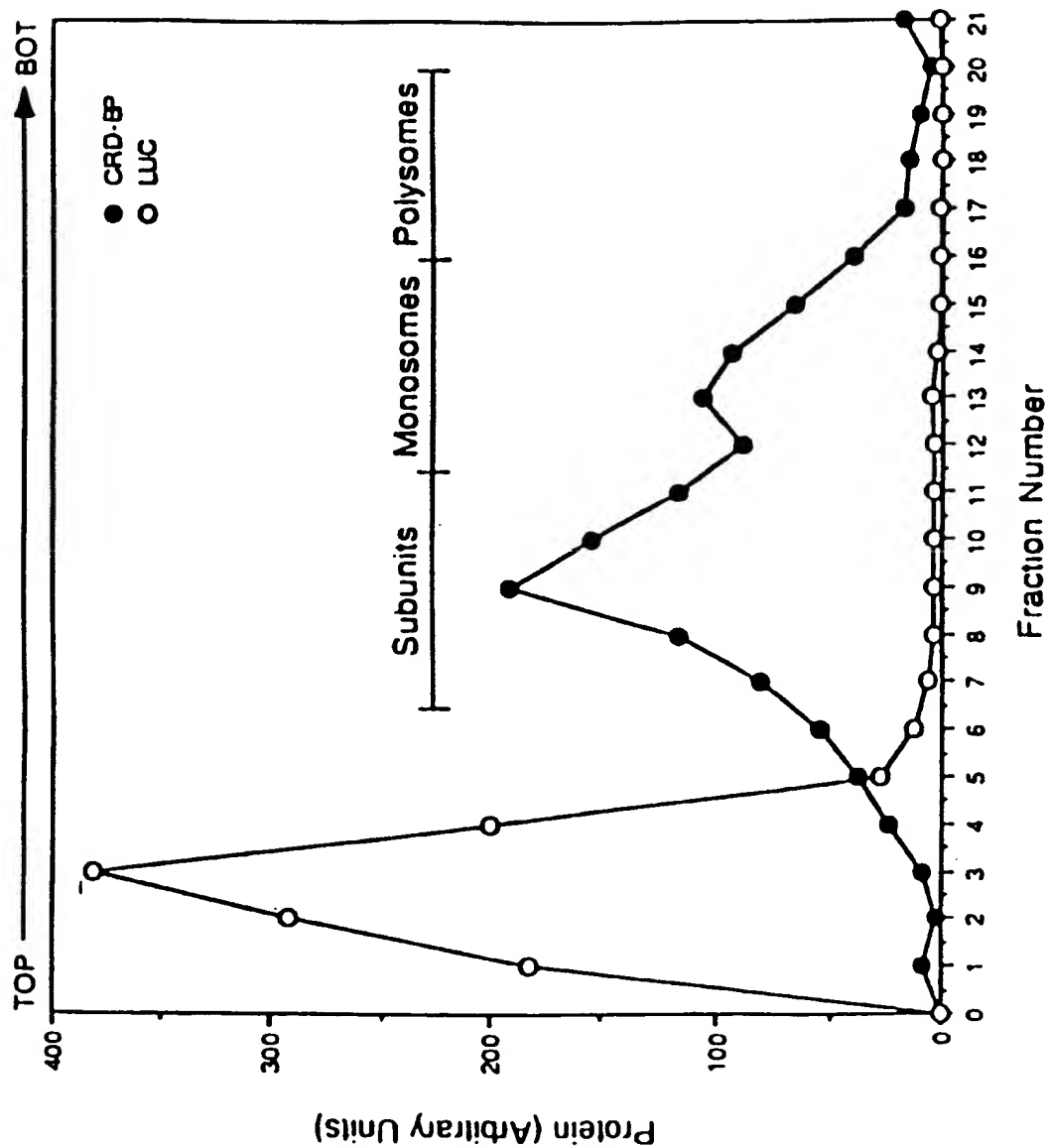


FIG. 4A



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FIG. 6A

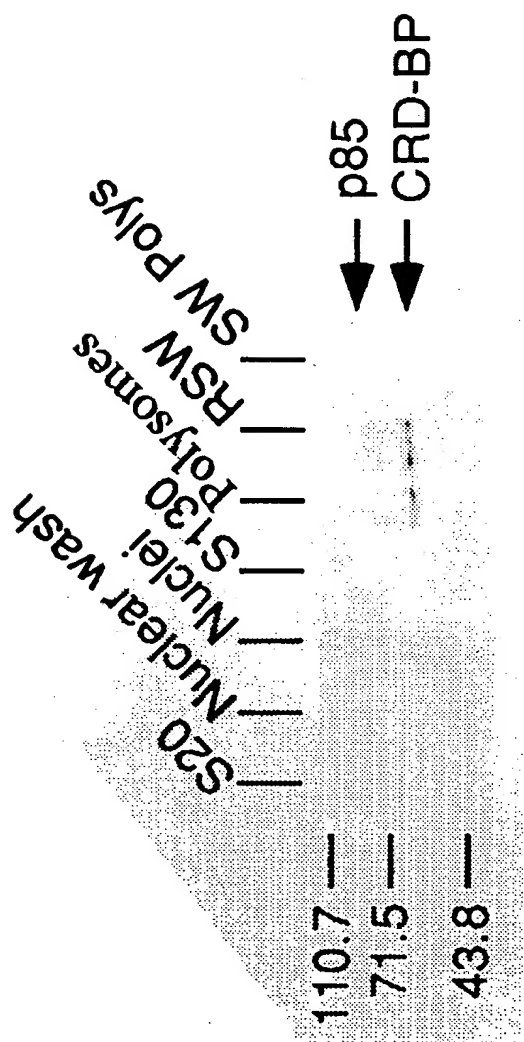


FIG. 6B



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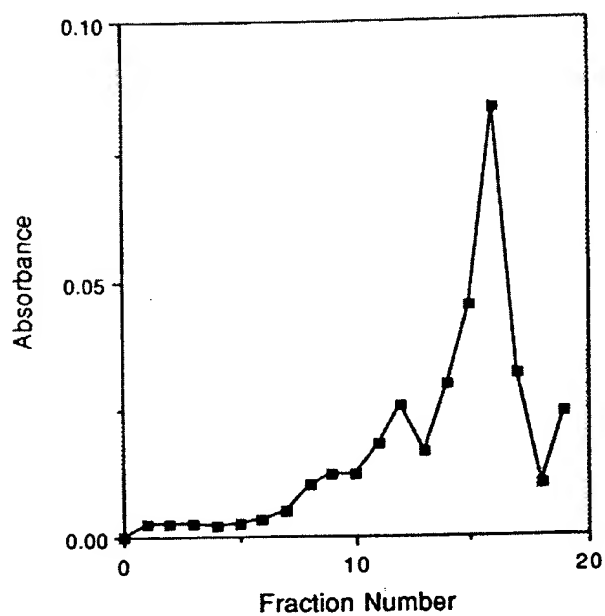


FIG. 7A

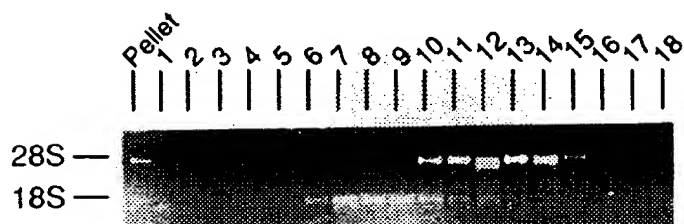


FIG. 7B

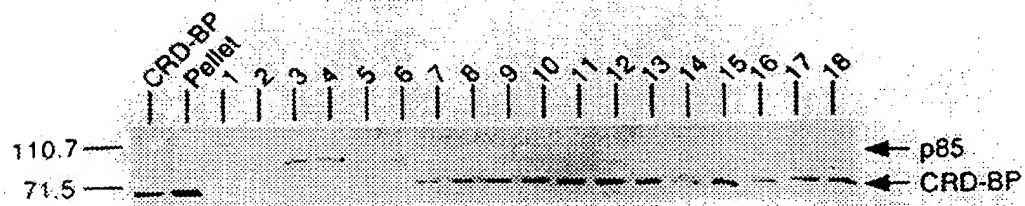


FIG. 7C

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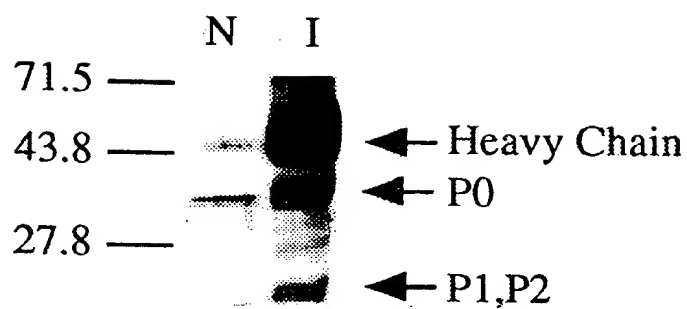


FIG. 8A

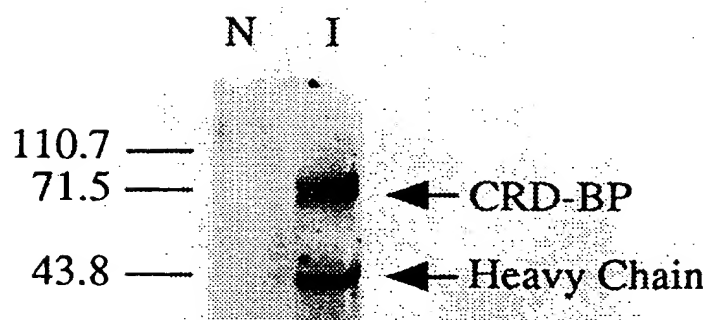


FIG. 8B

## SEQUENCE LISTING

&lt;110&gt; Ross, Jeffrey

<120> THE C-MYC CODING REGION DETERMINANT-BINDING PROTEIN  
(CRD-BP) AND ITS NUCLEIC ACID SEQUENCE

&lt;130&gt; 960296.95131

&lt;140&gt;

&lt;141&gt;

&lt;160&gt; 46

&lt;170&gt; PatentIn Ver. 2.0

&lt;210&gt; 1

&lt;211&gt; 2224

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 1

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&lt;210&gt; 2

&lt;211&gt; 577

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 2

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Met Asn Lys Leu Tyr Ile Gly Asn Leu Asn Glu Ser Val Thr Pro Ala
 1          5          10          15

Asp Leu Glu Lys Val Phe Ala Glu His Lys Ile Ser Tyr Ser Gly Gln
      20          25          30

Phe Leu Val Lys Ser Gly Tyr Ala Phe Val Asp Cys Pro Asp Glu His
      35          40          45

Trp Ala Met Lys Ala Ile Glu Thr Phe Ser Gly Lys Val Glu Leu Gln
      50          55          60

Gly Lys Arg Leu Glu Met Glu His Ser Val Pro Lys Lys Gln Arg Ser
      65          70          75          80

Arg Lys Ile Gln Ile Arg Asn Ile Pro Pro Gln Leu Arg Trp Glu Val
      85          90          95

Leu Asp Ser Leu Leu Ala Gln Tyr Gly Thr Val Glu Asn Cys Glu Gln
      100          105          110

Val Asn Thr Glu Ser Glu Thr Ala Val Val Asn Val Thr Tyr Ser Asn
      115          120          125

Arg Glu Gln Thr Arg Gln Ala Ile Met Lys Leu Asn Gly His Gln Leu
      130          135          140

Glu Asn His Ala Leu Lys Val Ser Tyr Ile Pro Asp Glu Gln Ile Thr
      145          150          155          160

Gln Gly Pro Glu Asn Gly Arg Arg Gly Gly Phe Gly Ser Arg Gly Gln
      165          170          175

Pro Arg Gln Gly Ser Pro Val Ala Ala Gly Ala Pro Ala Lys Gln Gln
      180          185          190

Pro Val Asp Ile Pro Leu Arg Leu Leu Val Pro Thr Gln Tyr Val Gly
      195          200          205

Ala Ile Ile Gly Lys Glu Gly Ala Thr Ile Arg Asn Ile Thr Lys Gln
      210          215          220

Thr Gln Ser Lys Ile Asp Val His Arg Lys Glu Asn Ala Gly Ala Ala
      225          230          235          240

Glu Lys Ala Ile Ser Val His Ser Thr Pro Glu Gly Cys Ser Ser Ala
      245          250          255

Cys Lys Met Ile Leu Glu Ile Met His Lys Glu Ala Lys Asp Thr Lys
      260          265          270

Thr Ala Asp Glu Val Pro Leu Lys Ile Leu Ala His Asn Asn Phe Val
      275          280          285

Gly Arg Leu Ile Gly Lys Glu Gly Arg Asn Leu Lys Lys Val Glu Gln
      290          295          300

Asp Thr Glu Thr Lys Ile Thr Ile Ser Ser Leu Gln Asp Leu Thr Leu
      305          310          315          320

Tyr Asn Pro Glu Arg Thr Ile Thr Val Lys Gly Ala Ile Glu Asn Cys
      325          330          335

Cys Arg Ala Glu Gln Glu Ile Met Lys Lys Val Arg Glu Ala Tyr Glu
      340          345          350

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Asn Asp Val Ala Ala Met Ser Leu Gln Ser His Leu Ile Pro Gly Leu  
 355 360 365  
 Asn Leu Ala Ala Val Gly Leu Phe Pro Ala Ser Ser Ser Ala Val Pro  
 370 375 380  
 Pro Pro Pro Ser Ser Val Thr Gly Ala Ala Pro Tyr Ser Ser Phe Met  
 385 390 395 400  
 Gln Ala Pro Glu Gln Glu Met Val Gln Val Phe Ile Pro Ala Gln Ala  
 405 410 415  
 Val Gly Ala Ile Ile Gly Lys Lys Gly Gln His Ile Lys Gln Leu Ser  
 420 425 430  
 Arg Phe Ala Ser Ala Ser Ile Lys Ile Ala Pro Pro Glu Thr Pro Asp  
 435 440 445  
 Ser Lys Val Arg Met Val Val Ile Thr Gly Pro Pro Glu Ala Gln Phe  
 450 455 460  
 Lys Ala Gln Gly Arg Ile Tyr Gly Lys Leu Lys Glu Glu Asn Phe Phe  
 465 470 475 480  
 Gly Pro Lys Glu Glu Val Lys Leu Glu Thr His Ile Arg Val Pro Ala  
 485 490 495  
 Ser Ala Ala Gly Arg Val Ile Gly Lys Gly Gly Lys Thr Val Asn Glu  
 500 505 510  
 Leu Gln Asn Leu Thr Ala Ala Glu Val Val Val Pro Arg Asp Gln Thr  
 515 520 525  
 Pro Asp Glu Asn Asp Gln Val Ile Val Lys Ile Ile Gly His Phe Tyr  
 530 535 540  
 Ala Ser Gln Met Ala Gln Arg Lys Ile Arg Asp Ile Leu Ala Gln Val  
 545 550 555 560  
 Lys Gln Gln His Gln Lys Gly Gln Ser Asn Leu Ala Gln Ala Arg Arg  
 565 570 575

Lys

<210> 3  
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 <212> PRT  
 <213> Mus musculus

<400> 3  
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 1 5 10

<210> 4  
 <211> 14  
 <212> PRT  
 <213> Homo sapiens

<400> 4  
 Gly Arg Arg Gly Leu Gly Gln Arg Gly Ser Ser Arg Gln Gly  
 1 5 10

<210> 5  
<211> 14  
<212> PRT  
<213> Homo sapiens

<400> 5  
Gly Arg Gly Gly Phe Asp Arg Met Pro Pro Gly Arg Gly Gly  
1 5 10

<210> 6  
<211> 13  
<212> PRT  
<213> Homo sapiens

<400> 6  
Gly Arg Gly Gly Phe Gly Asp Arg Gly Gly Arg Gly Gly  
1 5 10

<210> 7  
<211> 14  
<212> PRT  
<213> Homo sapiens

<400> 7  
Gly Arg Gly Gly Phe Gly Gly Arg Gly Gly Gly Arg Gly Gly  
1 5 10

<210> 8  
<211> 14  
<212> PRT  
<213> Homo sapiens

<400> 8  
Leu Arg Arg Gly Asp Gly Arg Arg Arg Gly Gly Gly Arg Gly  
1 5 10

<210> 9  
<211> 13  
<212> PRT  
<213> Artificial Sequence

<400> 9  
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1 5 10

<210> 10  
<211> 11  
<212> PRT  
<213> Mus musculus

<400> 10  
Gln Leu Arg Trp Glu Val Leu Asp Ser Leu Leu  
1 5 10

<210> 11  
<211> 11  
<212> PRT  
<213> Homo sapiens



<400> 11  
His Leu Gln Trp Glu Val Leu Asp Ser Leu Leu  
1 5 10

<210> 12  
<211> 10  
<212> PRT  
<213> Homo sapiens

<400> 12  
Gln Leu Arg Leu Glu Arg Leu Gln Ile Asp  
1 5 10

<210> 13  
<211> 11  
<212> PRT  
<213> Homo sapiens

<400> 13  
Thr Ile Ser Ser Leu Gln Asp Leu Thr Leu Tyr  
1 5 10

<210> 14  
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<213> Homo sapiens

<400> 14  
Thr Ile Ser Pro Leu Gln Glu Leu Thr Leu Tyr  
1 5 10

<210> 15  
<211> 11  
<212> PRT  
<213> Human immunodeficiency virus

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Gln Leu Pro Pro Leu Glu Arg Leu Thr Leu Asp  
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1 5

<210> 17  
<211> 47  
<212> PRT  
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Ala Thr Ile Arg Asn Ile Thr Lys Gln Thr Gln Ser Lys Ile Asp Val  
20 25 30

His Arg Lys Glu Asn Ala Gly Ala Ala Glu Lys Ala Ile Ser Val  
35 40 45

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<213> Mus musculus
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```
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Ile Leu Ala His Asn Asn Phe Val Gly Arg Leu Ile Gly Lys Glu Gly
  1             5             10             15
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Arg Asn Leu Lys Lys Val Glu Gln Asp Thr Glu Thr Lys Ile Thr Ile  
20 25 30

Ser Ser Leu Gln Asp Leu Thr Leu Tyr Asn Pro Glu Arg Thr Ile Thr  
35 40 45

**Val**

```
<210> 19
<211> 47
<212> PRT
<213> Mus musculus
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Val Phe Ile Pro Ala Gln Ala Val Gly Ala Ile Ile Gly Lys Lys Gly  
1 5 10 15

Gln His Ile Lys Gln Leu Ser Arg Phe Ala Ser Ala Ser Ile Lys Ile  
20 25 30

Ala Pro Pro Glu Thr Pro Asp Ser Lys Val Arg Met Val Val Ile  
35 40 45

```
<210> 20
<211> 48
<212> PRT
<213> Mus musculus
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```
<400> 20
Ile Arg Val Pro Ala Ser Ala Ala Gly Arg Val Ile Gly Lys Gly Gly
  1                      5          10          15
```

Lys Thr Val Asn Glu Leu Gln Asn Leu Thr Ala Ala Glu Val Val Val  
20 25 30

Pro Arg Asp Gln Thr Pro Asp Glu Asn Asp Gln Val Ile Val Lys Ile  
35 40 45

```
<210> 21
<211> 47
<212> PRT
<213> Homo sapiens
```

<400> 21  
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1 5 10 15

Ala Thr Ile Arg Asn Ile Thr Lys Gln Thr Gln Ser Lys Ile Asp Val  
20 25 30

```
<400> 22
Ile Leu Ala His Asn Asn Pro Val Gly Arg Leu Ile Gly Lys Glu Gly
  1                      5              10              15
```

Ser Pro Leu Gln Glu Leu Thr Leu Tyr Asn Pro Glu Arg Thr Ile Thr  
35 40 45

<400> 23  
Gln Phe Ile Pro Ala Leu Ser Val Gly Ala Ile Ile Gly Lys Gln Gly  
1 5 10 15

Ala Pro Ala Glu Ala Pro Asp Ala Lys Val Arg Met Val Ile Ile  
35 40 45

<400> 24  
Ile Arg Val Pro Ser Phe Ala Ala Gly Arg Val Ile Gly Lys Gly Gly  
1 5 10 15

Pro Arg Asp Gln Thr Pro Asp Glu Asn Asp Gln Val Val Val Lys Ile  
35 40 45

<400> 25  
Ile Leu Leu Gln Ser Lys Asn Ala Gly Ala Val Ile Gly Lys Gly Gly  
1 5 10 15

Lys Asn Ile Lys Ala Leu Arg Thr Asp Tyr Asn Ala Ser Val Ser Val  
20 25 30

```

<400> 29
Ile Gln Val Pro Arg Asn Leu Val Gly Lys Val Ile Gly Lys Asn Gly
  1          5          10          15
Lys Leu Ile Gln Glu Ile Val Asp Lys Ser Gly Val Val Arg Val Arg
          20          25          30

```

Ile Glu Ala Glu Asn Glu Lys Asn Val Pro Gln  
35 40

<210> 30  
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<213> Artificial Sequence

<400> 30  
Leu Leu Val Gly Leu Ile Gly Lys Gly Gly Leu Lys Leu Leu Leu Arg  
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Ile Ile

<210> 31  
<211> 17  
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<213> Artificial Sequence

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<210> 32  
<211> 17  
<212> DNA  
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<400> 32  
ggvacvacva cytcdbg 17

<210> 33  
<211> 17  
<212> DNA  
<213> Artificial Sequence

<400> 33  
gctgccgtca aattctg 17

<210> 34  
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<212> DNA  
<213> Artificial Sequence

<400> 34  
tcgacggttt ccatatg 17

<210> 35  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<400> 35  
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<210> 36  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<400> 36  
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<210> 37  
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<400> 37  
aaccggctc gagcggccgc t 21

<210> 38  
<211> 24  
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<400> 39  
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<210> 40  
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<212> DNA  
<213> Artificial Sequence

<400> 40  
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<210> 41  
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<213> Artificial Sequence

<400> 41  
gactgcgtct gttttgtgat g 21

<210> 42  
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<212> DNA  
<213> Artificial Sequence

<400> 42  
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<210> 43  
<211> 32  
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<210> 44  
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20

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<223> Xaa where Xaa = Gln or Ile

<220>  
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<220>  
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<222> (11)  
<223> Xaa where Xaa = Ile or Lys

<220>  
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<223> Xaa where Xaa = Tyr or Gly

<220>  
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<222> (15)  
<223> Xaa where Xaa = Ile or Leu

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1 5 10 15

<210> 46  
<211> 14  
<212> PRT  
<213> Mus musculus

<400> 46  
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1 5 10

